

REMARKS/ARGUMENTS

Status of the Application

In the Non-Final Office Action mailed July 3, 2006, claims 2, 5-17, and 19 were rejected. In the present response, claims 5-14 and 19 were amended to remove dependencies from withdrawn claims. No new matter was added.

Rejections Under 35 U.S.C. § 103(a)

Claims 2, 5-17, and 19 were rejected under 35 U.S.C. § 103(a) as being obvious over Templeton *et al.*, Langmuir 15:66-76 (1999), in view of Foos *et al.*, Chem. Mater. 14:2401-08 (2002). Applicants respectfully traverse these rejections.

By citing *In re Kerkhoven*, 626 F.2d 846 (CCPA 1980), for the premise that “[i]t is *prima facie* obvious to combine two compositions each of which is taught by the prior art to be useful for the same purpose, in order to form a third composition to be used for the very same purpose,” the Examiner apparently believes that the Applicants’ claimed invention is a trivial combination of a capture coating component and a shielding component on a metallic nanoparticle. The non-trivial nature of making mixed monolayer protected Au particles containing oligo ethylene glycol, however, is in the difficulty of mapping out conditions for the synthesis. The water content in the reaction solvent mixture has to be well-controlled in order to get the product with good yield. This control of water content was a key finding that had never been disclosed in the prior art. Applicants discussed this point in their 2003 JACS paper, which is attached hereto as Exhibit 3. Note that Foos *et al.* is cited in this 2003 JACS paper, evidencing the fact that the referees of this leading chemical journal recognized the significance of Applicants’ work even though it was presented to the journal almost a year after Foos *et al.*’s online publication date.

In any event, Applicants attach herewith a 131 Declaration signed by Ming Zheng that swears behind the online publication date of Foos *et al.* (April 19, 2002). With the removal of Foos *et al.* as a reference, Applicants respectfully submit that the claimed invention is nonobvious over Templeton *et al.*

SUMMARY

In view of the foregoing remarks, Applicants submit that this application is in condition for allowance. In order to expedite disposition of this case, the Examiner is invited to contact either of Applicants' representatives at the telephone numbers listed below to resolve any remaining issues. Should there be a fee due which is not accounted for, please charge such fee to Deposit Account No. 04-1928 (E.I. du Pont de Nemours and Company).

Respectfully submitted,

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Ethylene Glycol Monolayer Protected Nanoparticles for Eliminating Nonspecific Binding with Biological Molecules[†]

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Nanoparticles and their hybrid with biological molecules are recognized to have potential applications in electronics, optics, genomics, proteomics, and biomedical and bioanalytical areas.^{1–6} The usefulness of the hybrid materials in many occasions depends on how well one can achieve rational design based on specific binding between inorganic nanomaterials and biological molecules. It is generally recognized that the surfaces of many metallic nanoparticles, such as Au, Ag, Pt, and Cu, are charged,⁷ which cause nonspecific binding with biological molecules via electrostatic interactions. Alkanethiol protected nanoparticles reduce the electrostatic interactions, but induce hydrophobic interactions. In addition, alkanethiol protected nanoparticles are not soluble in water, which makes them noncompatible with biomolecules. Nonspecific binding between nanoparticles and biomolecules is a fundamental issue that is not well addressed in the published literature.⁸

In this paper, we demonstrate a new approach to eliminate nonspecific interactions between nanoparticles and proteins by synthesizing ethylene glycol protected gold nanoparticles. Ethylene glycol type molecules are water soluble and neutral in charge and have been demonstrated to resist protein binding on flat gold surfaces.⁹ These early successes prompted us to use ethylene glycol type molecules for nanoparticles. However, the synthesis of an ethylene glycol monolayer on a flat gold surface is very different from that on nanoparticles. Two examples have been demonstrated for preparing poly(ethylene glycol) (PEG) protected gold nanoparticles.¹⁰ It is well understood that poly(ethylene glycol) chains form random coils on a nanoparticle surface. When a ligand is introduced onto the surface of the nanoparticle, the ligand can be easily shielded by the polymer coils, preventing programmable assembling with biological molecules. The ideal surface structure should be a densely packed ethylene glycol monolayer with a well-defined length that is inert to biological binding. In the meantime, a functional ligand can be introduced so that it is accessible to targets, such as protein, cell, etc., for specific binding. To achieve this goal, a short chain with a well-defined length is preferred to long polymer chains for coating nanoparticles.

Very recently, Foos et al. reported the synthesis of short ethylene oxide chain protected gold nanoparticles by the ligand exchange reaction.¹¹ The procedure involved the synthesis of hexanethiol (C6) protected gold nanoparticles followed by two steps of replacing C6 with ethylene glycol thiol molecules. There was also a report on the direct synthesis of tetra(ethylene glycol) protected gold nanoparticles in the aqueous phase with a very low molar ratio of reducing agent (NaBH₄) and tetrachloroauric acid, resulting in partial reduction.¹² With an increasing amount of NaBH₄, the reaction increased aggregation. The yield is very low. Here, we report a direct synthesis of ethylene glycol (HS-(CH₂CH₂O)_n-CH₃, n = 2, 3, and 4) protected gold nanoparticles in a mixed solvent of methanol and water by utilizing the NaBH₄ reduction

method, with much improved yield.¹³ The key for this synthesis is the control of water content in the reaction mixture. It was found that water content from 9% to 18% (v/v) is optimized for the formation of stable and water-soluble nanoparticles.

Di-, tri-, and tetrachethylene glycol thiol molecules (HS-(CH₂CH₂O)_n-CH₃, n = 2, 3, and 4) were synthesized and purified in our lab.¹⁴ Synthesis of di-, tri-, and tetra(ethylene glycol) monolayer protected gold nanoparticles (Au-S-EG₂, Au-S-EG₃, and Au-S-EG₄) was achieved by controlling the addition of water into the reaction mixture.¹⁴ When the water content was either higher than 18% or lower than 9%, the reaction mixtures began to form agglomerates. It was also noticed that the reaction mixture became aggregated if the reagents contained a very small amount of impurity, such as HS-(CH₂CH₂O)_n-OH (n = 2, 3, or 4). The mechanism for the critical water concentration was not investigated. When diluted, these directly synthesized ethylene glycol protected gold nanoparticles became red/purple and clear. The directly synthesized ethylene glycol gold nanoparticles were found to be very stable in aqueous solution and could withstand high salt concentration (1 M NaCl). After 9 months of storage in aqueous solution, no agglomeration was seen. Lyophilized particles could be readily redissolved in water to form a clear red/purple solution. In addition, Au-S-EG_n (n = 2, 3, or 4) was found to be very soluble in most organic solvents, such as acetone, methanol, DMSO, chloroform, DMF, and THF. In contrast, our experimental results showed that the indirectly synthesized Au-S-EG_n (n = 2, 3, or 4) nanoparticles¹³ by the replacement reaction with tiopronin protected gold nanoparticle (Au-Tp) could not be redissolved after centrifuging purification and drying, and the aggregation was also seen after storage in aqueous solution for several weeks.

The NMR spectra of both the free EG₄-SH molecule and the Au-S-EG₄ nanoparticle are attached in the Supporting Information. The disappearance of the -SH proton signal (t, 1.50–1.56 ppm) in the Au-S-EG₄ nanoparticle clearly supports that the gold nanoparticle is bonded with the EG₄-S- monolayer. The chemical shifts and the integrals of the rest protons confirm that the bonded ethylene glycol molecules have the same structure as the free ones. The average size of the nanoparticles is about 3 nm, as confirmed by the TEM measurement.¹⁴

Protein binding to Au nanoparticles can be conveniently monitored by gel electrophoresis, because the protein–nanoparticle complex is expected to migrate differently than the free Au particles. Protein binding reactions were done by mixing 10 μ L of ~0.1 mM Au particles with 1 μ L of 10 mg/mL protein in sodium phosphate buffer (50 mM, pH 7.3). After incubation at room temperature for 10 min, the entire reaction mixture was loaded on a 1% agarose gel.¹⁴ Gel electrophoresis results are shown in Figure 1. When Au-Tp particles were mixed with lysozyme and BSA, different degrees of nonspecific binding were observed, as indicated by the band shifts. When the same experiment was done with Au-S-EG₄ particles, no change in the particle mobility was observed. These

[†] Publication No. 8392.

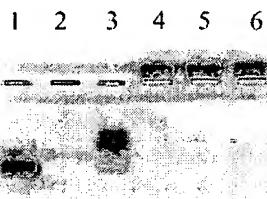


Figure 1. Gel image of protein binding assay. Eleven microliters of protein/particle binding reaction mixture was loaded onto a 1% agarose gel, and electrophoresis was run for 20 min at 90 V constant voltage. Lane 1: 10 μ L of Au-Tp at \sim 100 μ M. Lanes 2 and 3 are the same amount of Au particles mixed with 1 μ L of lysozyme and BSA, respectively. Protein concentrations are all 10 mg/mL. Lanes 4–6 are similar to lanes 1–3, except that Au-EG₄ particles were used.

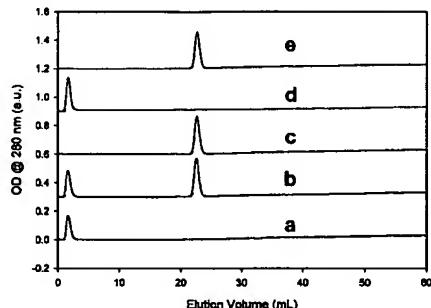


Figure 2. Strong cation exchange chromatography for Au-Tp and Au-EG₄ binding with lysozyme. (a) Au-EG₄; (b) Au-EG₄ + lysozyme; (c) lysozyme; (d) Au-Tp; and (e) Au-Tp + lysozyme.

results clearly showed that the Au-S-EG₄ does not bind to either the very basic protein lysozyme or the very acidic protein BSA.

The most dramatic contrast was provided by the binding between Au particles and lysozyme. Addition of lysozyme into the Au-Tp particle solution causes an immediate color change from pinkish red to blue, indicating that Au particles form aggregates, presumably because the positively charged lysozyme ($pI = 11$) molecules cross-link negatively charged Au-Tp particles ($-COOH$). Centrifugation of the Au-Tp/lysozyme reaction mixture resulted in a clear/colorless supernatant, indicating that no soluble Au-Tp particles remained in the supernatant. The cation exchange column chromatography confirmed that only lysozyme proteins were present in the supernatant, as shown in Figure 2. However, when lysozyme was mixed with Au-S-EG₄ solutions, no color change happened in the solution or the supernatant after centrifugation of the Au-EG₄/lysozyme mixture. The reaction mixture was eluted from the cation exchange column as two separate species corresponding to lysozyme and Au-S-EG₄ particles. This result indicates that there is no binding reaction occurring between Au-S-EG₄ and the lysozyme protein. Also tested were cytochrome C, ribonuclease A, and myoglobin. In all cases, no binding was observed between Au-S-EG₄ and the tested proteins, but a varying degree of binding between Au-Tp and those proteins was seen. The Au-EG₄ nanoparticle was also run through an anion exchange column. The Au-S-EG₄ nanoparticles were eluted at the void volume from both cation and anion exchange columns, indicating that the surface of Au-S-EG₄ particles is charge neutral and does not bind to either

column. Consistent with this, we also found that Au-S-EG_n particles do not bind to either total cellular RNA or chromosomal DNA, both of which are highly negatively charged.¹⁴

In conclusion, we discovered that with the water content optimized in the range of 9–18% in the reaction mixture, di-, tri-, and tetra(ethylene glycol) protected gold nanoparticles Au-S-EG_n ($n = 2, 3$, and 4) could be directly synthesized. These gold nanoparticles that are bonded with a uniform monolayer with defined length varying from 0.8 to 1.6 nm (from molecular modeling) have great stability in aqueous solutions with a high concentration of electrolyte and organic solutions. Using ion-exchange chromatography and gel electrophoresis, we first demonstrated that these Au-S-EG_n ($n = 2, 3$, or 4) nanoparticles with neutral and hydrophilic surfaces have complete resistance to protein nonspecific interactions. These types of nanoparticles provide a fundamental starting material for designing hybrid materials composed of metallic nanoparticles and biomolecules. One of the advantages for the direct synthesis method is that it allows the synthesis of a mixed monolayer of ethylene glycol and a functional ligand to eliminate nonspecific interactions and provide specific interactions in the same time.¹⁵ From the aspect of industrial applications, these biologically inert gold nanoparticles will have commercial utilities in electronic and biomedical applications. Some of these works are underway.

Supporting Information Available: Synthesis of EG_n-SH ($n = 2, 3$, or 4), synthesis of the Au-EG₄ nanoparticle, synthesis of Au-S-EG_n ($n = 2, 3$, or 4) through replacement with the tiopronin protected Au nanoparticle, gel electrophoresis experiment, ion exchange chromatography, ¹H NMR spectra of free EG₄-SH and the Au-S-EG_n nanoparticle, TEM image of Au-EG₄, and gel electrophoresis assay of Au-EG₄ binding with DNA and RNA (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- (14) See Supporting Information.
- (15) Manuscript in preparation.

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